

Final Report

Grant #: N00014-92-J-1612

1993

R & T 213f006

Principal Investigator: Barbara A. Araneo, Ph.D.Institution: University of Utah, Pharmadigm, Inc.Grant Title: Preservation Of Immunologic Responsiveness Following TraumaAward period: 1 April 1992 - 31 December 1996

OBJECTIVE: To question whether the acute administration of intravenous DHEA or DHEAS prevents reduction in host resistance that develops in mice following an experimental hemorrhagic shock. To determine whether the acute administration of intravenous DHEA prevents the development of bacterial translocation after hemorrhagic shock in mice.

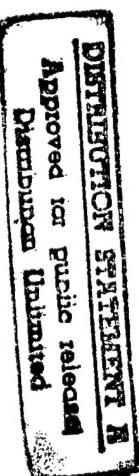
APPROACH: We propose to investigate the detrimental effects of hemorrhagic shock on immunocompetence, and then to question whether acute administration of intravenous DHEA at the time of resuscitation preserves host resistance and immune function. Our approach will be to study the immunomodulatory effects of shock and resuscitation. Mice are maintained under deep anesthesia while an amount of blood is removed to depress blood pressure to near shock. After two hours at low blood perfusion, mice are resuscitated with lactated Ringers. The formula for resuscitation is the removed blood plus two times the volume of shed blood as Ringers. Either DHEA or vehicle are added to the Ringers solution. Measures of oxidant potential in tissues, acute phase cytokines and mean arterial pressure were used to establish the acute prophylactic benefits of DHEA.

ACCOMPLISHMENTS: The results of extensive studies have demonstrated that mice subjected to hemorrhagic shock and resuscitation suffer sever oxidative stress as evidenced by a smaller ratio of reduced to oxidized glutathione, higher levels of serum IL-6, and increased incidence of bacterial translocation. Further if bacterial translocation is facilitated by cecal ligation after the resuscitation phase, there is a higher incidence of mortality. In contrast, mice treated with DHEA at the time of resuscitation, possess more oxidant potential in the liver and other organs, produce less IL-6 and are less susceptible to the bacterial translocation or the effects of facilitated translocation. This work has contributed in a meaningful way to support clinical trials testing the efficacy of DHEA in the management of acute inflammation.

As a result of our final efforts to investigate the effect of DHEA on epithelial barrier function. We conducted several preliminary studies on epidermal barrier function. Using pigs, epidermal barrier function was destroyed by excising the skin to 0.008" in sections measuring 5 x 5 cm. on the dorsal trunk using a dermatome. Re-epithelialization of the sites on placebo versus DHEA treated pigs was monitored by histologic evaluation.

Restoration of epidermal barrier function was assessed in the two treatment regimens by change in skin conductance using a dermal phase meter. The results showed a statistically significant acceleration (30% increase) in the time to re-epithelialization and restoration of barrier function. We believe we will be able to show this same effect on mucosal epithelium. Work ongoing in our labs will explore the effect of DHEA on gut barrier function in vivo, and then on intestinal epithelial cell barrier function in vitro.

SIGNIFICANCE: It is well recognized that individuals who are recovering from trauma possess an increased risk for life-threatening infections. It is known that these forms of physical stress induce imbalances in both glucocorticoid and DHEA production. We propose that the changes in steroid metabolism, generally seen in patients with severe thermal injury or recovering from surgical trauma, are largely responsible for mediating the pronounced and protracted deficit in immune function and host resistance linked to these



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stress conditions. Further, we believe that therapeutic intervention with DHEA designed to correct stress-induced changes in steroid hormone levels will also result in a preservation of normal immune function and host resistance.

Work Plan: Research for the remainder of the funding period will focus on alterations in host resistance and oxidative stress in a shock/surgery model in rodents. First, we propose to continue our investigation of hemorrhagic shock on gut barrier function, susceptibility to endotoxin challenge and oxidative potential in the liver and other more sensitive organs like the lung..

PUBLICATIONS:

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- 4) R Lohman, R Yowell, S Barton, B Araneo, M Siemionow. 1997. DHEA protects muscle flap microcirculatory hemodynamics from ischemia/reperfusion injury (an experimental *in vivo* study). J trauma: Injury, Infection, and Critical Care 42:74-80
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Dehydroepiandrosterone Protects Muscle Flap Microcirculatory Hemodynamics from Ischemia/Reperfusion Injury: An Experimental In Vivo Study

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This study evaluated the potential for dehydroepiandrosterone (DHEA) to protect skeletal muscle from reperfusion injury using intravital microscopic observations of isolated rat cremaster muscle flaps. The flaps were subjected to warm ischemia followed by reperfusion in three groups of rats. In group 1 (control, n = 14), muscle flaps were subjected to 6 hours of ischemia and then evaluated after either 90 minutes (n = 8) or 24 hours (n = 6) of reperfusion. Group 2 animals (propylene glycol pretreatment, n = 8) were pretreated with a propylene glycol vehicle, then underwent 6 hours of ischemia and were evaluated after 90 minutes reperfusion. Group 3 animals (DHEA pretreatment, n = 12) were pretreated with DHEA dissolved in propylene glycol, subjected to 6 hours of ischemia, and then evaluated after either 90 minutes (n = 6) or 24 hours (n = 6) of reperfusion.

Red blood cell velocity in the flap's main arteriole, functional

capillary density, venular constriction index (the ratio of internal to external diameter of postcapillary venules), and microemboli formation were measured. Muscle samples were evaluated by electron microscopy.

Control animals showed a 61% reduction in red blood cell velocity ($p < 0.05$) accompanied by a 69% reduction in functional capillary density ($p < .05$) acutely and total cessation of flow by 24 hours. No differences between control and propylene glycol treated animals were noted. In DHEA-pretreated animals, reflow occurred in 100% of the flaps, there was a temporary 39% reduction ($p < 0.05$) in functional capillary density, and all flaps remained viable at 24 hours.

In this study, DHEA pretreatment markedly improved muscle flap microcirculatory hemodynamics and protected flaps against ischemia/reperfusion injury.

METHODS

There is scientific evidence suggesting that cellular damage after prolonged ischemia occurs during tissue reperfusion.¹⁻⁴ Upon reperfusion, a cascade of events takes place, including the generation of toxic oxygen species and inflammatory mediators, leading to activation, adherence, and transmigration of polymorphonuclear neutrophils, and ultimately resulting in local tissue damage, flow compromise, and subsequent failure of the microcirculation.⁵⁻⁹ Different agents and means of microcirculatory protection have been used in the past, however none have proved to be routinely useful in replantation surgery or emergency free flaps.

Dehydroepiandrosterone (DHEA) is a naturally occurring, weakly androgenic, catabolic steroid known to have diverse effects when administered in pharmacologic doses.¹⁰⁻¹⁴ It can prevent progressive ischemic damage in burn models by preserving vascular function in thermally injured tissue.¹⁵ Here we report on the use of DHEA to modify microcirculatory hemodynamic events after ischemia/reperfusion injury. To study its effect on flow return at the capillary level, a rat cremaster muscle flap model for intravital microscopy was used, since hemodynamic changes with functional capillary perfusion can easily be evaluated with this preparation.

Thirty-four male, inbred Sprague-Dawley rats weighing 130 to 180 gm were assigned to one of three experimental groups. *Group 1 (n = 14) control:* After isolation of the neurovascular pedicle, the cremaster flap was subjected to 6 hours of ischemia followed by reperfusion. Eight animals were evaluated in the 90 minutes immediately after reperfusion and an additional 6 animals were evaluated 24 hours after reperfusion. *Group 2 (n = 8) propylene glycol pretreatment:* Animals were first subcutaneously injected with 0.20 mL of propylene glycol (Sigma Chemical Company, St. Louis, Mo) into the fat pad of the left groin at 24 hours, and again at 1 hour before the onset of ischemia. The animals were then subjected to 6 hours of ischemia and evaluated over 90 minutes after reperfusion. *Group 3 (n = 12) DHEA pretreatment:* 4.0 mg/kg of DHEA dissolved in 0.20 mL of propylene glycol (both from Sigma) was subcutaneously injected into the fat pad of the left groin at 24 hours, and again at 1 hour before the onset of ischemia. Next, animals were submitted to 6 hours of clamp ischemia and evaluated at 90 minutes (n = 6) and 24 hours (n = 6) after reperfusion.

This operative protocol complies with animal use standards accepted by the Institutional Animal Care and Use Committee of the University of Utah. All animals were anesthetized with pentobarbital (50–60 mg/kg) intraperitoneally.

Surgical Dissection of the Cremaster Muscle Flap

The right cremaster muscle was mobilized and prepared in the following manner: The common femoral and external

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iliac vessels were isolated and all branches, with the exception of the pudic-epigastric that supplies the cremaster flap, were ligated. This allows for total vascular isolation of the flap. The cremaster muscle was then separated from the overlying scrotal skin and Cooper's fascia was stripped away. After removal of the testicle, the empty tube of cremaster muscle was fully mobilized, its pedicle consisting of the pudic-epigastric vessels, and a small strip of external oblique muscle was left intact.

Protocol for Microcirculatory Measurements

The rat was then placed on a specially designed plexiglas stage, lactated Ringer's solution (Baxter, McGaw Park, Ill) was supplied at a rate of 2 mL/hour intraperitoneally and core temperature was maintained at $36^\circ \pm 1^\circ\text{C}$ for the duration of the experiment. The flap was then opened ventrally with thermal cautery and suspended in a tissue bath that allowed transillumination for intravital microscopy. Observations were made using a Nikon Optiphot-2 microscope equipped with a closed-circuit television camera (Hitachi KP-C503) and a final magnification of $1800\times$ on a video monitor (Sony Trinitron). The system was also fitted with a custom laser Doppler (Texas A&M Microcirculation Research Institute, College Station, Tex) to measure red blood cell (RBC) velocity.

For sampling purposes, three postcapillary venules were selected, one in each of the proximal, middle, and distal portions of the flap. The density of functional capillaries was estimated by counting the number of perfused capillaries in nine high-power field windows around each of the preselected postcapillary venules. Thus, a total of 27 windows were screened, the average of which is presented as the density of perfused capillaries for the entire flap. The ratio of the internal to external diameter of the three postcapillary venules was also calculated and is presented as the lumen constriction index. This factor is an important measure of vascular injury, because a decrease in the internal diameter relative to the external diameter of postcapillary venules occurs during endothelial swelling. RBC velocity of the flap's main artery (A1) was also measured by optical Doppler velocimeter.

Baseline measurements were made in all animals after the cremaster flap was placed on the tissue bath. To submit the cremaster flap to ischemia, microvascular clamps were applied to the external iliac and common femoral arteries and veins just proximal and distal to the origin of the pudic-epigastric vessels (Fig. 1). After 6 hours, the clamps were released and measurements were made at 30, 60, and 90 minutes later. During the acute phase experiments, animals remained under general anesthesia for the entire period of observation. For animals evaluated at 24 hours, the cremaster tube was preserved in a subcutaneous tunnel created at the medial border of the right hind limb. These animals were allowed to recover from anesthesia and later reanesthetized for evaluation.

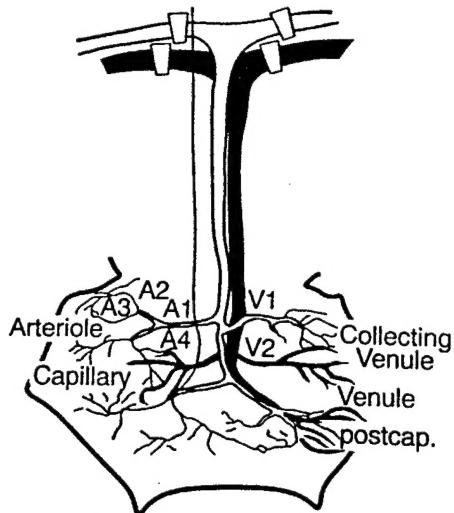


FIG 1. Schematic representation of the cremaster muscle flap model isolated on the pudic-epigastric pedicle. Vascular clamps are applied to the iliac artery and vein just above and below the origin of the pedicle.

Histology

Cremaster samples were harvested 90 minutes and 24 hours after reperfusion for electron microscopy. Portions of the muscle mass were preserved in Karnofsky fixative for ultrastructural examination. Tissue was postfixed in osmium, dehydrated in graded acetones, and embedded in epoxy resin. Ultrathin sections from selected tissue blocks were stained with lead citrate and uranyl acetate. Specimens were interpreted by a pathologist blinded to the study.

Statistical Evaluation

Data was analyzed with SPSS-PC software (SPSS, Inc., Chicago, Ill). One-way analysis of variance was used for intergroup comparison, while the Student's *t* test was used for intragroup comparison. The *p* value of <0.05 was considered significant.

RESULTS

Group 1: Control

In control animals, reflow after 6 hours of ischemia occurred initially in all eight animals. However, flow stopped in one animal after 30 minutes, and in another, after 60 minutes. None of the flaps were perfused in the six animals evaluated at 24 hours. RBC velocity in the A1 arteriole dropped from a baseline level of 35.5 ± 2.4 mm/s to 11.9 ± 2.6 mm/s ($p < 0.05$) 30 minutes after clamp release and further decreased to 6.5 ± 2.0 mm/s after 90 minutes. By 24 hours, there was *no flow observed* in the A1 arteriole (see Fig. 2). The density of perfused capillaries followed a similar pattern: 30 minutes after clamp release, the number of functional capillaries dropped from a baseline level of $12.4 \pm 0.3/\text{hpf}$ (high power field) to $3.9 \pm 0.6/\text{hpf}$ ($p < 0.05$). At 24 hours, *no functional capillaries* were seen (see Fig. 3). The lumen constriction index was reduced from a baseline level of 0.85 ± 0.01 to 0.78 ± 0.09 ($p < 0.05$) 30 minutes after clamp release, and remained at this level for 90 minutes. By 24 hours, perfusion failure made it impossible to identify

A1 VELOCITY

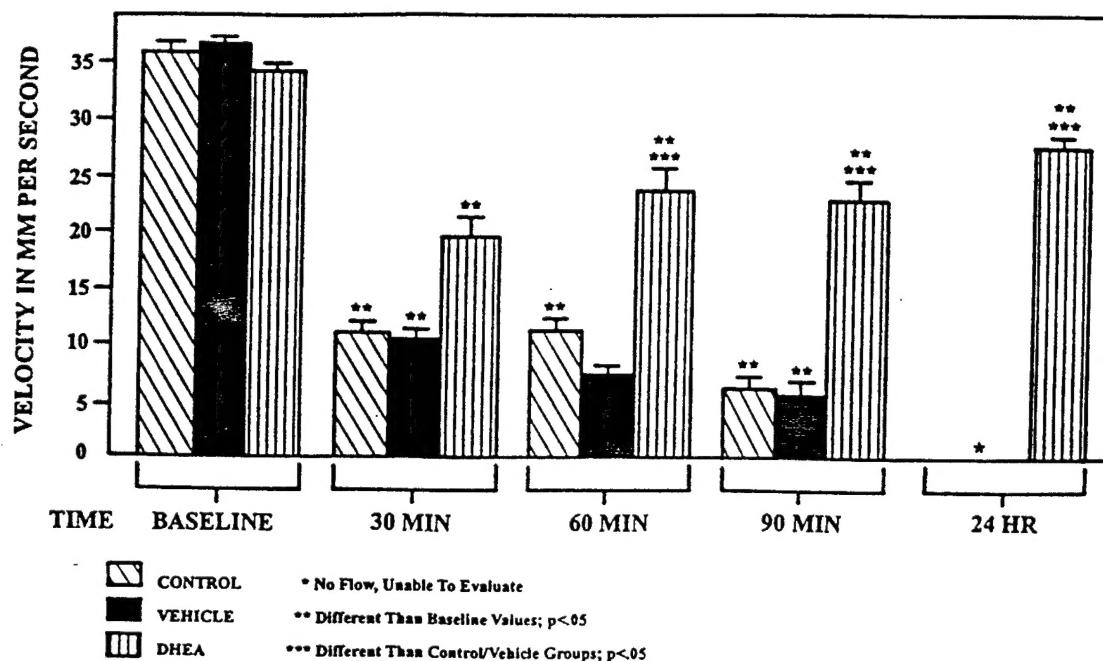


FIG 2. Comparison of the A1 RBC flow velocity between baseline, preischemic values and at the various times after clamp release. Animals underwent 6 hours of warm ischemia after the baseline data were collected. In control animals, there was no prograde flow in the A1 arteriole at 24 hours.

FUNCTIONAL CAPILLARY DENSITY

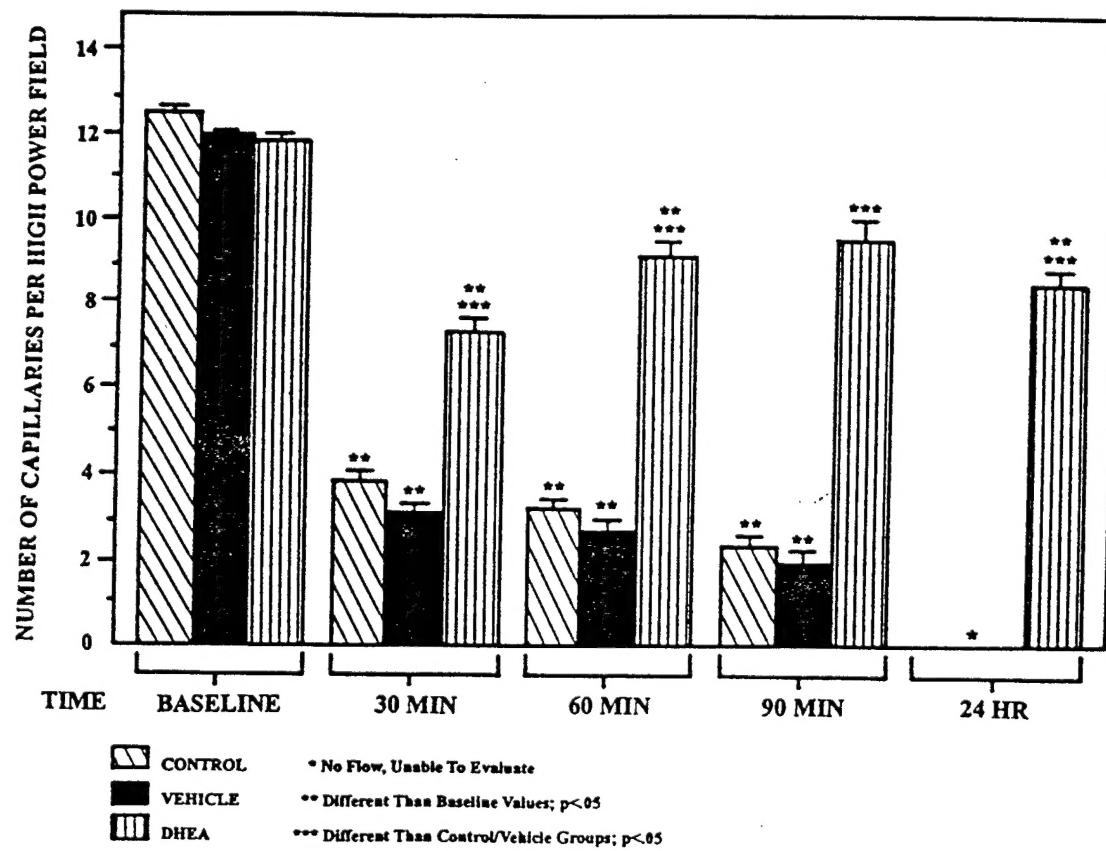


FIG 3. Comparison of functional capillary density, as represented by the average number of flowing capillaries per hpf, between preischemic values and at various time points after 6 hours of warm ischemia. In control animals, there were no functional capillaries 24 hours after clamp release.

TABLE 1. Lumen constriction index

	Control (Group 1)	Vehicle (Group 3)	DHEA (Group 3)
Baseline	0.85/0.01 (n=8)	0.84/0.02 (n=8)	0.86/0.04 (n=6)
30 minutes	0.78/0.07 (n=8) ^a	0.79/0.04 (n=7) ^a	0.84/0.02 (n=6)
60 minutes	0.79/0.04 (n=7) ^a	0.78/0.03 (n=7) ^a	0.86/0.05 (n=6) ^b
90 minutes	0.78/0.09 (n=6) ^a	0.79/0.02 (n=6) ^a	0.84/0.01 (n=6) ^b
24 hours	No flow (n=6) ^a		0.83/0.01 (n=6) ^b

^a Different than baseline, $p < 0.05$.

^b Different than baseline, $p < 0.05$.

Comparison of the constriction index (inner diameter/outer diameter) of the postcapillary venules between baseline values and at various times after 6 hours of warm ischemia. At 24 hours, it was not possible to identify postcapillary venules in the control animals.

postcapillary venules, thus constriction index could not be measured (see Table 1).

Group 2-Propylene Glycol Pretreatment

In animals pretreated with propylene glycol, initial reflow occurred in seven out of eight flaps and, after 60 minutes, six out of eight flaps were perfused. At no point did any of the measured parameters differ significantly between the control and propylene glycol groups.

Group 3: DHEA Pretreatment

In animals pretreated with DHEA, reflow occurred in all six flaps immediately after clamp release, and six out of six flaps were well perfused at 24 hours.

The A1 RBC velocity in *Group 3* dropped from 34.5 ± 1.1 mm/s to 19.9 ± 4.4 mm/s ($p < 0.05$) 30 minutes after clamp release. These values, however, began to rise, reaching a maximum of 27.3 ± 1.8 mm/s at 24 hours. Capillary perfusion dropped from an initial level of $11.9 \pm 0.3/\text{hpf}$ to $7.3 \pm 0.8/\text{hpf}$ ($p < 0.05$) after 30 minutes of reflow. At 90 minutes after clamp release, the number of functional capillaries *returned to baseline levels*: $11.9 \pm 0.3/\text{hpf}$ versus $9.6 \pm 0.9/\text{hpf}$ (p , not significant), which was never observed in the control groups. The constriction index did not change during the experiment (see Table 1).

Histology

Photomicrographs of normal nonischemic muscle and of representative sections from each of the control and treatment groups are compared in Figures 4–8.

Figure 4 depicts plentiful, normal mitochondria in a nonischemic muscle. A bands are organized with numerous electron-dense granules representing glycogen. Figures 5 and 6 are photomicrographs comparing high power views of mitochondria contained in vehicle-treated and DHEA-treated muscle after 6 hours of ischemia and 90 minutes of reperfusion. The representative section in Figure 5 illustrates degeneration of the mitochondria, the absence of glycogen, and frank edema of the cremaster muscle in vehicle-treated rats. In contrast, the condition of the cremaster muscle from DHEA-treated rats (Fig. 6) is markedly improved and viable. Mitochondria remain intact with a distinctive presence of glycogen, and much less edema. After 6 hours of ischemia and 24 hours of reperfusion, the cremaster muscle of vehicle-treated rats (Fig. 7) shows little evidence of intact mitochondria and extensive vacuolization of the muscle. This supports

the findings reported in Figures 2 and 3 demonstrating no reflow in the muscle at 24 hours. In Figure 8, the cremaster muscle of the DHEA-treated rats after 6 hours of ischemia and 24 hours of reperfusion contains numerous mitochondria. In these sections, the myofibrils are compact with little ede-

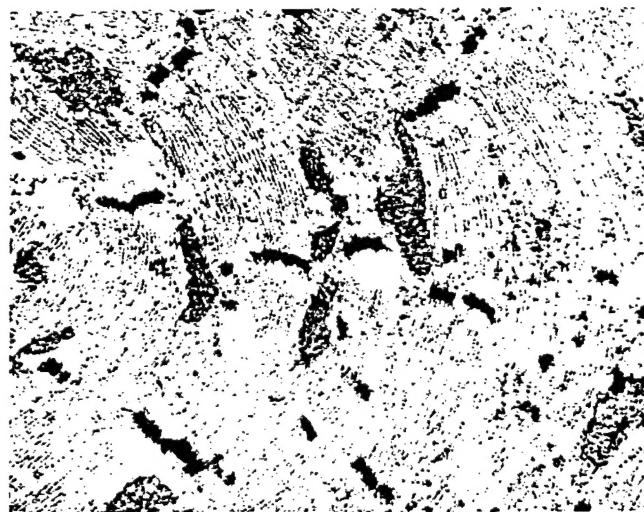


FIG 4. Transmission electron microscopy (EM) of normal nonischemic rat cremaster muscle at 37,000 \times . Plentiful, normal mitochondria and well-organized muscle bands are apparent. Dense granules represent glycogen.

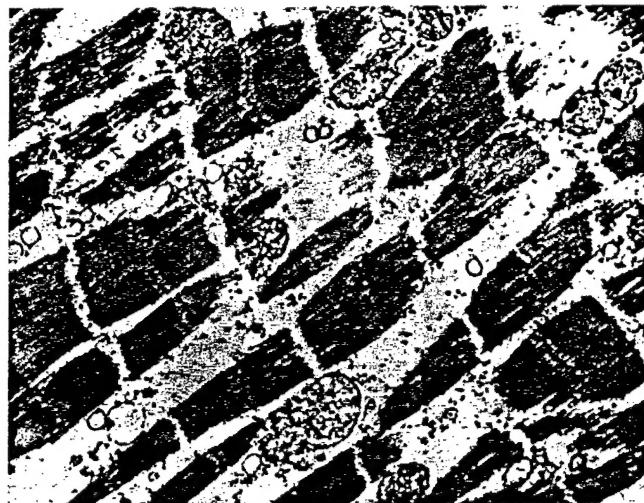


FIG 5. Transmission EM of cremaster muscle pretreated with propylene glycol vehicle after 6 hours of ischemia and 90 minutes of reperfusion at 25,000 \times . Degeneration of the mitochondria, frank edema, and absence of glycogen are evident.



FIG 6. Transmission EM of cremaster muscle pretreated with DHEA after 6 hours of ischemia and 90 minutes of reperfusion at 25,000 \times . Preserved mitochondria, minimal muscle edema, and presence of glycogen are seen.

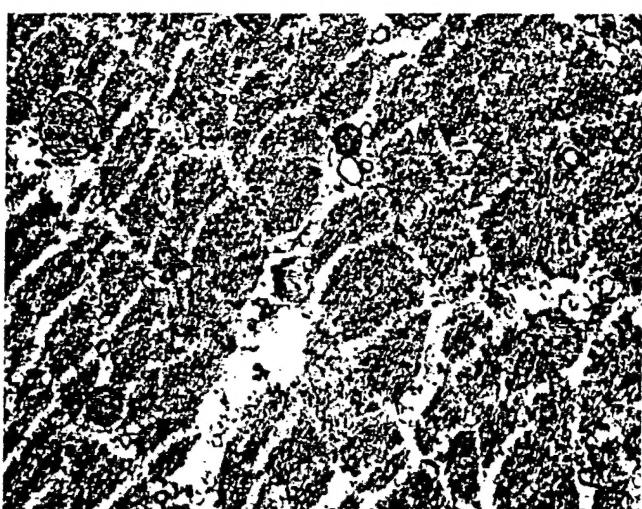


FIG 8. Transmission EM of cremaster muscle pretreated with DHEA after 6 hours of ischemia and 24 hours of reperfusion at 25,000 \times . Numerous mitochondria are present, however muscle is somewhat depleted of glycogen.



FIG 7. Transmission EM of cremaster muscle pretreated with propylene glycol vehicle after 6 hours of ischemia and 24 hours of reperfusion at 20,000 \times . Degenerative mitochondria and extensive muscle vacuolization are evident.

ma; however the muscle appears somewhat depleted of glycogen.

DISCUSSION

We have chosen the isolated rat cremaster muscle model for this study because it allows direct *in vivo* observation of capillary perfusion, changes in postcapillary venule size, and RBC flow velocity. Furthermore, these parameters are known to remain constant at normal levels for at least 3 days in cremaster flaps not subjected to ischemia/reperfusion injury.¹⁶ None of the changes reported here can be explained by the passage of time alone. After total vascular isolation of the flap, complete arrest of blood flow is achieved by microvascular clamp application. In this way, crush injury is avoided and no collateral flow is possible, both of which can be problems in tourniquet ischemia models.

In control animals, 6 hours of warm ischemia resulted initially in minimal reflow, followed by a visible decline in flap perfusion and cessation of flow at 24 hours. This proved to be lethal to the muscle flaps and is consistent with the

observations of other authors.^{2,17,18} As expected, propylene glycol had no effect on microcirculatory hemodynamics. Animals pretreated with DHEA demonstrated almost normal flow dynamics, with 100% flow return after both 90 minutes and 24 hours of reperfusion. Swelling of the endothelium was not observed and RBC velocity was maintained at 77% of normal at 24 hours. This was continued by electron microscopy, where only slight evidence of damage to myocytes was seen.

The pathogenesis of tissue damage after prolonged ischemia is complex and involves both "no reflow" occurrence and reperfusion injury.^{5,6} The "no reflow" phenomena occurs when there is a failure of perfusion in the capillary bed despite return of flow in the major feeding vessels. The end result of both phenomena is local tissue death, as seen in both the control and vehicle-pretreated groups. DHEA intervention preserved vascular function, possibly by maintaining endothelial integrity after ischemia/reperfusion injury, leading to improved tissue survival. Araneo et al. have noted that treatment with DHEA, and not any of its metabolites, results in preservation of vascular architecture and function after thermal injury.¹⁵

The "no reflow" phenomenon has been ascribed to a variety of mechanisms, including microvascular thrombosis and endothelial swelling. Quinones-Baldrich et al. proposed microvascular thrombosis as an explanation for perfusion failure.¹⁹ Jesse et al.²⁰ have shown that DHEA inhibits arachidonic acid-induced platelet aggregation both *in vitro* and *in vivo*. However, histologic study by others has not identified evidence of platelet or fibrin thrombi in muscles injured by reperfusion.^{4,19,21} Platelet aggregates or fibrin thrombi interfering with capillary flow were not observed during intravital microscopic observations in this study. This is in agreement with other authors who have proved that treatment with anticoagulant agents does not improve the "no reflow problem."^{4,22,23} It seems unlikely that DHEA preserves capillary function after ischemia/reperfusion injury through antiplatelet adhesion or anticoagulation actions.

Mazzoni observed endothelial cell swelling after ischemic/reperfusion injury and has suggested that this luminal narrowing leads to increased resistance in the microvascular bed, thereby contributing to the "no reflow" phenomenon.^{24,25} In the present study, we observed endothelial swelling in the postcapillary venules (decreased lumen constriction index) was observed in control and vehicle-treated animals, but there was no evidence of endothelial edema in the animals protected with DHEA. This may explain the improved capillary perfusion seen in these animals. The histologic data showing compact myofibrils and numerous mitochondria in muscle samples pretreated with DHEA supports these observations.

The inflammatory reaction caused by ischemia/reperfusion injury leads to the production of oxygen radicals that are toxic to vascular endothelium. Mohan has reported on the ability of DHEA to inhibit superoxide generation²⁶ and this may be part of the mechanism by which DHEA protects vascular function after reperfusion. Thromboxane also regulates events after reperfusion injury and agents that block thromboxane production, such as indomethacin, have been shown to improve tissue perfusion after thermal injuries.^{27,28} DHEA may also be acting to interfere with either thromboxane production or its effects.

In this study, there was a direct correlation between the physical measurements used to evaluate the hemodynamics of the microcirculation and the histologic vitality of the muscle. We have presented evidence that the DHEA treatment sustains near normal hemodynamics within a 90-minute reflow analysis. Additionally, there is convincing data that, because of DHEA treatment, muscle structure and capillary functional integrity remain intact over 24 hours of reperfusion. One additional explanation for the preservation of capillary perfusion may be the significant decrease in the number of adherent and transmigrating leukocytes that we have observed after DHEA pretreatment.* These findings are in agreement with Korthuis et al., as well as with our previous study, where increased muscle viability was attributed to the depletion of leukocytes from the microcirculation.^{29,30} DHEA is known to have a wide range of effects, acting as an anti-inflammatory agent, an antiplatelet agent, antioxidant, and immunoregulator. These, or some undiscovered effect, perhaps play a role in its ability to protect against reperfusion injury. At least in the experimental setting, DHEA administration appears safe and may be of value as a presurgical prophylactic agent to reduce the effects of reperfusion injury. In the clinical setting of free tissue transfer, where ischemic time is often critical, DHEA pretreatment may be useful because of its beneficial effect in the microcirculation. Replantation surgery might also benefit from DHEA use if an intravenous vehicle, capable of achieving rapid tissue levels or use as a perfusate for the amputated part, could be developed. As more is discovered about its mechanism of action, DHEA may become an important clinical tool.

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